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Progress during the reporting period included: 1) Immunization of mice with dengue serotype 1 (DEN-1) structural proteins and a modified form of NS1 expressed in insect cells using a recombinant baculovirus. 2) Immunization of mice with an antigenic subfragment of the DEN-1 E protein expressed in *E. coli*. 3) Demonstration that the E protein fragment produced in *E. coli* is capable of eliciting virus neutralizing antibodies when injected with alumina as the adjuvent. 4) Generation of large clones of DEN-1 cDNA using the polymerase chain reaction to amplify reverse transcripts of viral RNA. 5) Determination of experimental conditions for the efficient transfection of BHK cells in culture with purified DEN-1 genomic RNA. 6) Development of a sensitive RNA protection assay to determine the ratio of plus- and minus-strand viral RNA in DEN-1-infected cells.

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FOREWORD

For the protection of human subjects, the investigator(s) have adhered to policies of applicable Federal Law 45CFR46.

In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

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Annual Report - DAMD 17-86-C-6156
15 June 1989 - 14 September 1990

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A. Other Personnel Comprising the Project Team.

The contract personnel are listed below. In the absence of a continuation contract, we severely reduced the size of the research team during the contract period. In addition, full-time, highly trained personnel left the project and were replaced with less experienced and/or part-time workers

R. Huebner, Ph.D. Postdoctoral Fellow	6/88 to 7/90
C. Thomas, Ph.D. Postdoctoral Fellow (part-time)	5/90 to present
A. Semproni, M.S. Research Assistant	6/86 to 7/89
L. Smith, B.S. Technician	1/90 to present
D. Wysokenski, B.A. Graduate Assistant	1/89 to 2/91

B. Project Aims:

The primary goals for the reporting period are listed below. This report details progress made during the year encompassing 15 June 1989 through 14 September 1990. The original award period was 15 June 1986 to 14 June 1989. The award was extended without additional funding to 14 September 1990.

1) Development of DEN-1 and JE subunit vaccines. Emphasis has been divided between *E. coli* host-vector expression systems and production of recombinant DEN-1 E and NS1 immunogens in insect host cells using recombinant viruses of the *Autographica californica* nuclear polyhedrosis virus (baculovirus).

2) Identification and assembly of large clones of DEN-1 cDNAs.using PCR amplification of viral RNA.

3) Define conditions to measure the infectivity of DEN-1 genomic RNA and derivatives synthesized *in vitro*.

4) Develop sensitive hybridization assays to determine the ratios of plus- and minus-stranded RNAs in DEN-1-infected insect cells.

C. Program:

Evaluation of antibodies generated against recombinant DEN-1 immunogens

Antisera produced against recombinant E domain-II proteins from *E. coli*, delta-34 and delta-34 cys-to-ser, were tested for their ability to neutralize DEN-1 virus in an in vitro PRNT assay. None of the antisera tested were found to neutralize virus in this assay. Antisera produced against unpurified extracts of cells infected with a recombinant baculovirus expressing DEN-1 proteins were also tested in this assay and no virus neutralizing antibodies were detectable. The recombinant baculovirus (BR-7) used to make these extracts expressed all the DEN-1 structural proteins and a modified form of the NS1 protein which is missing the last 70 carboxyl-terminal amino acids.

The antisera made against extracts of cells infected with the BR-7 were also evaluated by immunoblotting. These analyses revealed that antibodies could be detected which reacted with authentic E and NS1 proteins from both C6/36 and BHK cells.

Evaluation of recombinant DEN-1 immunogens

During this period, several experiments were performed to test the immunogenecity of recombinant DEN-1 antigens injected into mice. The immunogens were either derivatives of the DEN-1 E protein (spanning amino acids 293-401) produced in *E. coli* or proteins produced in insect cells infected with the recombinant baculovirus BR-7 in which the DEN-1 coding sequences for C, prM, M, E and a truncated form of NS1 lacking the last 70 carboxyl-terminal amino acids are under the control of the baculovirus polyhedrin promoter. One of the proteins expressed in *E. coli*, designated Δ34-Ser, is a mutant form in which the cysteine residue at position 285 of the E protein sequence has been replaced by a serine. For immunization, the proteins were either bound to alumina or injected as an emulsion with Freund's complete adjuvent. Typically, the animals were immunized on days 0, 14 and 28 of the experiment, and ascitic fluids were generated by injection of sarcoma cells on day 35.

The sera from these animals were characterized for anti-DEN-1 antibody titers in plaque reduction neutralization tests, western blot analysis, hemagglutination inhibition and ELISA. The results for for the PRNT assays are given in Table 1.

Table 1. Plaque reduction neutralization titers for mouse antisera generated by immunization with recombinant DEN-1 proteins.

ANTIGEN (Adjuvent)	ANIMAL	PRNT
BR-7 (Alumina)	1	1:10
	2	1:20
	3	1:5
	4	nd
	5	1:5
BR-7 (CFA)	1	1:5
	2	nd
Δ34-SER (Alumina)	1	nd
	2	1:20
	3	nd
	4	nd
	5	nd
	6	nd
	7	nd
Δ34-CYS (Alumina)	1	1:40
	2	1:40
	3	nd
	4	1:40
	5	1:20
	6	1:20
	7	1:40
	8	1:80

[1] Dilution of antiserum yielding greater than 50% plaque reduction
[2] nd = no plaque reduction observed at 5-fold dilution

The data in Table 1 are significant for two reasons. First, the recombinant antigen produced in bacteria elicited virus neutralizing antibodies. The PRNTs for the Δ34-cys were 5- to 10-fold lower than the values obtained with ascitic fluids generated with hybridoma cells secreting the neutralizing monoclonal antibody D2-9D12 (data not shown). We believe these results represent the first demonstration of

dengue neutralizing antibodies generated with a recombinant antigen produced in *E. coli*. Second, alumina appears to offer significant advantages over CFA for generating neutralizing antibodies with recombinant antigens. It should be noted that in several previous immunization trials, over a period of two years, we were unable to detect neutraliztion titers in any of the animals immunized with recombinant antigens in CFA. The basis of the beneficial effect of alumina is not obvious. However, one possibility is that adsorption of the protein to the surface of alumina particles is an effective way of presenting the antigen in the "correct" conformation. The results also show that the removal of cysteine residue 285 in the E protein sequence actually decreased the effectiveness of the Δ34 antigen. We originally thought that the presence of cysteine-285 might interfere with the formation of the essential disulfide bridge between cysteines 302 and 333 in the E protein sequence (cf. Mason *et al.* 1989 and 1990).

The antisera to the Δ34 were reactive in western blots against the authentic E protein from DEN-1 infected cells, but the titer of these sera was very low in hemagglutination inhibition assays (little or no inhibition at 1:5 dilution). For comparison, the HAI titer for the D2-9D12 monoclonal antibody was >1/4096.

Identification and assembly of large clones of DEN-1 cDNAs using PCR amplification of viral RNA

Recombinant DNA technology is being employed to examine the antigenic potential of recombinant proteins produced in prokaryotic and eukaryotic expression systems. A logical progression in the use of genetic engineering in vaccine development is the production of recombinant attenuated viral strains. This can be achieved through the construction of full-length DEN-1 cDNA for transcription into infectious RNA molecules

The strategy developed for the production of full-length DEN-1 cDNA involves the use of the polymerase chain reaction to amplify segments of the genome that can be ligated together and then transcribed *in vitro* to produce infectious RNA. To date, two fragments (fragments #2 and 3) representing sequence information from nucleotides 2580-7235 have been amplified and cloned into pBluescript SK$^-$. Fragment #1 containing the beginning of the DEN-1 genome and the XhoI site at nucleotide 2579 was amplified but has not yet been successfully cloned into the Bluescript vector. This fragment was designed to contain an SP6 promotor for use in the production of full-length transcripts when the *in vitro* ligated DEN-1 cDNA becomes available. Preliminary data obtained from an *in vitro* transcription assay using a template containing a 20-nucleotide

dengue consensus sequence suggests that the SP6 promotor may function efficiently in the context of the surrounding sequences. A T3 promotor might be more efficient in producing full-length transcripts, and this will be tested in future studies.

Amplification of the 3'-segment encoding nucleotides 7260 to the 3' end was not successful by the PCR strategies employed. This may indicate failure of the consensus primers to initiate first strand synthesis. Two possible reasons for this are: 1) the secondary structure at the 3' end of DEN-1 RNA might inhibit first strand synthesis; 2) the consensus sequence used to design primers at the 3' end may differ from the actual sequence of the DEN-1 RNA. Future work may require determining the nucleotide sequence at the 3' end of the viral RNA.

Optimization of conditions to measure the infectivity of DEN-1 genomic RNA and derivatives synthesized *in vitro*.

The evaluation of *in vitro*-synthesized RNAs requires an efficient procedure for transfection of the RNA into susceptible host cells. To this end, we have been optimizing conditions for transfection with DEN-1 RNA extracted from virions. Prior to this study, the only flavivirus nucleic acid used for transfection experiments was yellow fever RNA (Rice *et al*, 1989). The results of the present study indicate that DEN-1 RNA can be successfully transfected into cultured cells. This procedure should make it possible to assay the infectivity of transcripts prepared both *in vivo* and *in vitro*.

The following conditions were used to obtain efficient transfection of BHK-21-15 cells with DEN-1 RNA. Approximately 1.5 x 10^5 cells in a 177 mm^2 area (one well of a standard 24 well tissue culture plate was transfected with 5 mg lipofectin and up to 1 ng full-length DEN-1 RNA in the presence of 500 ng yeast carrier tRNA. Thus, the lipofectin-RNA ratio was approximately 10:1. Lipofectin-RNA incubations were performed at 37°C with 500 ml Opti-MEM per well. An incubation time of seven hours resulted in transfection efficiencies in the range of 21-36 PFU/ng. This is approximately 5-30 fold less than the transfection efficiency obtained by Rice *et al* (1989) for yellow fever RNA. It may be that longer incubation times (up to 24 hours) would result in increased transfection efficiencies.

Development of sensitive hybridization assays to determine the ratios of plus- and minus-stranded RNAs in DEN-1-infected insect cells.

During the summer of 1990, we initiated experiments designed to accurately determine the relative levels of "plus" and "minus" strand DEN-1 transcripts present in infected C6/36 cells at various time-points, post-infection.

Total cellular RNA was extracted from cultures grown for 1, 2, 3, 4 or 5 days following infection with DEN-1. Various amounts of RNA, ranging from 0.1 μg to 50 μg, were hybridized to excess ^{32}P-labeled, *in vitro*-transcribed, 1-kb RNA probes complementary to either plus or minus DEN-1 transcripts. The hybridization reactions were subsequently treated with ribonuclease T1, which cleaves single-stranded RNA molecules at guanosine residues, but which does not recognize double-stranded RNA structures. Thus, probe that was hybridized to a plus- or minus-strand DEN-1 transcript was protected from enzymatic digestion. Separation of the reaction products on a denaturing polyacrylamide gel showed clearly that a single band, 1 kb in length, was protected from RNase T1 activity in both the "plus" and "minus" reactions. As ribonuclease protection assays (RPA) of this sort are performed using radiolabeled probes of known specific activity, the levels of the protected probes--and, by inference, levels of the corresponding DEN-1 transcripts--can be determined following separation of the reaction products on a denaturing polyacrylamide gel.

Our preliminary results indicate that, under the infection conditions used in this set of experiments, the ratio of plus-strand to minus-strand transcripts five days after initial infection of the culture is at least 10:1, and probably much higher. Extremely low levels of minus-strand transcripts in all of the samples made precise quantitation difficult.

While our results indicate that the RPA approach is potentially a useful method for quantification of *in vivo* levels of DEN-1 transcripts, several modifications to the infection protocol used in the preliminary experiments are required. First, as we wish to measure the relative levels of plus- and minus-strand transcripts at various time-points during the infectious cycle, it will be necessary to infect the cultures under conditions that ensure simultaneous infection of virtually all of the cells. Experiments performed subsequent to the RPA tests discussed above indicate that a 100% simultaneous infection rate requires a multiplicity of infection of approximately 10. To meet this requirement, we have prepared high-titer stocks of DEN-1 by two methods: concentration of existing low-titer stocks by ultracentrifugation; and by preparation of new stocks using a modified infection protocol. Second, we have refined our definition of early-, mid- and late-infection time-points by determining, via immunofluorescence, that DEN-1 antigens can be detected on the surface of 100% of the C6/36 cells 36 hours after infection at a high multiplicity of infection , whereas no immunofluorescence can be detected at 24 hrs post-infection. Thus, future RPA experiments will be performed on total cellular RNA extracted from cells infected at a high multiplicity of infection, at several time-points ranging from a few hours to 36 hours after infection. We believe that simultaneous infection of all cells in the culture will significantly improve the yield of

DEN-1 specific transcripts in total cell RNA preparations, thus solving the problem of low absolute levels of minus-strand RNA.

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E. Publications During the Reporting Period

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